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Markers of Rubella Virus Strains in RK_{13} Cell Culture

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When tested on RK_{13} cell cultures, strains of rubella virus could be differentiated by their ability to form small or large plaques. Large plaques were produced by the HPV-77 and Cendehill strains, and also by a laboratory stock strain (West Point), after only 14 passages in RK_{13} culture. Five wild-type rubella viruses, isolated and passaged only a few times in African green monkey kidney tissue culture, grew well in RK_{13} cell culture, but they were sensitive to agar inhibitors and, therefore, formed small plaques. On the other hand, RA27/3, an attenuated strain grown in WI-38 human fibroblast cells, developed low titers in RK_{13} cells and also produced small plaques. We concluded that the morphological differences between small-plaque and large-plaque viruses depended on their sensitivity to agar inhibitors and on the *p*H of the medium during plaque formation.

Many groups of animal viruses of the same antigenic type, notably polio-, myxo-, and poxviruses (1; G. D. Lawrence and A. Gould, 1968, Proc. 23rd Symp. Microbiol. Stand, *in press*), have been differentiated by in vitro markers. Rubella virus has been little investigated in this respect. Nevertheless, a number of attenuated strains have been developed, and vaccination is likely to reach the stage of large field trials (9, 10, 13, 14, 16) in the near future. We must, therefore, establish laboratory assay methods to distinguish various strains of attenuated and virulent rubella virus. In this paper, we describe marker characteristics in RK_{13} tissue culture by which rubella virus strains can be distinguished.

MATERIALS AND METHODS

Tissue cultures. RK_{13} cells (7) were grown in medium 199 with added antibiotics and 10% heat-inactivated fetal calf serum. The cells were maintained under a liquid overlayer with medium 199 and 2% fetal calf serum. Tylosin tartrate (Lilly) was added in a concentration of 40 μg/ml to growth or maintenance medium (3).

Virus strains. Various strains of rubella viruses were used. RA27/3 was isolated previously in this laboratory from an aborted fetus (12). In the marker tests, four pools of RA27/3 were used: (i) 8th passage level in WI-38 cell cultures; (ii) 25th passage level in WI-38 cell cultures (attenuated for man); (iii) 6 to 12th passage levels in BHK21 cells after eight passages in WI-38 cell cultures; and (iv) 15th passage level in RK_{13} cells, followed by plaque purification three times in the same cells. HPV-77 is an attenuated strain obtained from Parkman and Meyer (8, 9) and tested at the 78th passage level in African green monkey kidney (AGMK) cells, with or without subsequent plaque purification in RK_{13} cells. Cendehill is an attenuated strain obtained from Abel Prinzie (10), and tested at the 51st passage level in primary rabbit kidney cells. West Point is a laboratory stock strain passaged 14 times in RK_{13} cells, followed by three plaque purifications in the same cell line. We also used five low-passage level fresh isolates, obtained from the throats of adults with clinical rubella or infants with rubella syndrome, and tested at the second to fourth passage levels in AGMK cells (3). In addition, the Indiana strain of vesicular stomatitis virus (VSV), obtained from Werner Henle, was used in interferon studies.

Plaque technique. Plaques were produced in RK_{13} cells by techniques described previously (11). The solidifying agent used was 0.9% Noble's agar, 0.5% agarose (Seakem), or 0.75% carboxymethyl cellulose (Hercules Powder Co., Wilmington, Del.).

Plates overlaid with agar were incubated for 6 days in 3% CO_2 in air; a second overlayer was then added, containing the same substances plus a 1:8,000 concentration of neutral red. On day 7, these plates were examined for plaque formation. Plates overlaid with carboxymethyl cellulose media were left undisturbed for 7 days and then fixed and stained. A 5-ml portion of 10% formaldehyde was added to these plates; after 1 hr, the overlayer was removed by washing under water, and the monolayer was stained with 0.1% crystal violet solution.

Agar overlayers were used for production of plaques by VSV. After a 48-hr incubation period at 35 C in CO_2, plaques were stained with a 0.5% solution of neutral red and then counted.

A stock solution of 20 mg/ml of diethylaminoethyl (DEAE)-dextran (Pharmacia Inc., New Market, N.J.) was prepared in a saline solution, sterilized by filtration, and added to the overlayers in some experiments.

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Virus passage and plaque "purification." Maintenance medium for the preparation of stock viruses included tylosin to assure elimination of mycoplasma. Cloning of the virus, or plaque "purification," was performed by three successive passages of single plaques. In each step, the agar above one isolated plaque was excised and placed in 1 ml of maintenance medium; this was then used to inoculate other RK_{13} plates.

Tests for mycoplasma. The cells and virus pools were checked at intervals for mycoplasma by L. Hayflick. Before the addition of tylosin, the RK_{13} cells showed the presence of these contaminants. After several passages in the tylosin-containing medium, cells were consistently mycoplasma-free.

Growth curves. Confluent monolayers of RK_{13} cells were infected with 0.2 ml each of virus inoculum at the input multiplicities indicated in Results. After adsorption for 1 hr at 35 C in the CO_2 atmosphere, monolayers were washed three times, and 5 ml of maintenance medium was added to each plate. At the indicated intervals, replicate plates were removed from the incubator, their fluids were harvested, and the cells were removed by treatment with 0.25% trypsin in 0.02% Versene. Half of the extracellular fluid was stored for titration of virus, and half was set aside to test for the presence of interferon. The cell suspension was frozen and thawed three times, centrifuged at 165 × *g* for 10 min, and the supernatant fluid was titrated for virus.

Titrations of virus were determined by plaque assay on RK_{13} cells under carboxymethyl cellulose overlayers. In every growth-curve experiment, the virus pools used to initiate infection were tested simultaneously with the harvests to exclude variations in the results due to the sensitivity of small-plaque viruses to environmental conditions.

Titrations of interferon. Samples to be tested for the presence of interferon were treated with 1 N HCl and held overnight at 4 C at a *p*H of 2 (15). The *p*H was then made neutral by addition of 1 N NaOH.

Infectious virus was sedimented by centrifugation for 2 hr at 120,000 × *g* (Spinco model L centrifuge, no. 40 rotor). This procedure sedimented 99% of infective virus. For trypsin treatment of the interferon preparation, a 0.25% solution of trypsin was incubated for 1 hr at 37 C with an equal volume of undiluted tissue culture fluid.

To test for the presence of interferon, 2 ml of serial fourfold dilutions of the preparation was added to each replicate plate of RK_{13} cells. The interferon titer was expressed as the highest dilution of fluid which reduced the number of VSV plaques by 50%.

Antisera. Chinchilla rabbits (2 to 3 months old) were inoculated intravenously with 1 to 2 ml of undiluted virus suspension on alternate days for 2 weeks. A booster injection was given after an additional 2 weeks, and serum samples were obtained 6 days later.

Virus-containing fluids, harvested in the absence of fetal calf serum, were treated with 1 M sterile cobalt chloride to remove virus (4). Comparative titrations of supernatant fluid and sediment showed that 90 to 99% of the virus sedimented with the precipitate, which had a titer of $10^{6.5}$ plaque-forming units (PFU)/ml. Undiluted antisera were absorbed with the precipitated virus for 2 hr at 37 C; the precipitate was then removed by centrifugation.

Plaque reduction tests. The absorbed antisera were inactivated at 56 C for 30 min, destroying any remaining live virus. Viruses and antisera for the plaque reduction tests were diluted in medium 199 containing 20% normal rabbit serum. Each dilution of antiserum was incubated for 60 min at room temperature with a virus inoculum containing 30 to 50 PFU/0.2 ml. The neutralization tubes were shaken at 15-min intervals. Each virus-antiserum mixture was inoculated on three plates; the percentage of plaque survival was calculated in relation to a control virus sample incubated in the absence of antiserum.

RESULTS

Small-plaque and large-plaque variants: In initial experiments, two virus strains were compared: RA27/3 (eight passages in WI-38 cells, six passages in BHK21 cells), and West Point (15 passages in RK_{13} cells). Under an agar overlayer, the West Point plaques were relatively large and clear with well-defined boundaries, whereas the RA27/3 plaques were small and turbid. At day 7, the West Point plaques were approximately 3 mm in diameter; the RA27/3 plaques were only about 1 mm in diameter.

Differences were also noted in the kinetics of plaque formation. When plates were stained with neutral red 6 days after infection, the plaques of West Point virus were clearly discernible, whereas the RA27/3 plaques were not visible until the following day. In plates observed beyond day 7, West Point plaques increased further in diameter; plaques formed by RA27/3 virus not only failed to increase in size, but they frequently disappeared upon further incubation, becoming overgrown by normally stained cells.

To determine whether these morphological differences were caused by the adaptation of West Point strain to RK_{13} cells, RA27/3 virus was also passaged in RK_{13} cells. After 15 passages in RK_{13} cells, followed by three successive passages of isolated plaques, the RA27/3 virus continued to produce small plaques. In all subsequent experiments, unless otherwise stated, plaque-purified RA27/3 was used.

Among the rubella viruses tested were two other attenuated strains, HPV-77 and Cendehill 51. Both of these strains formed large plaques in RK_{13} cells. In contrast, the five freshly isolated rubella virus strains produced small turbid plaques.

The RA27/3 strain was also tested after 26 passages in WI-38 cell cultures and after 12 passages in BHK21 cells. The plaques produced were even smaller than those produced by RA27/3 passed in RK_{13} cells.

TABLE 1. *Influence of overlayer on plaque number and morphology of three rubella virus strains*[a]

Virus strain	Agar (0.9%)			Agar + 500 μg of DEAE-dextran/ml			Carboxymethyl cellulose		
	No.	D	Char	No.	D	Char	No.	D	Char
RA 27/3	60	2	T	81	3	C	90	2	T
HPV-77	62	3	C	68	4	C	60	2	C
H-600	5	1	T	47	2	T±	52	2	T

[a] Abbreviations: No., number of plaques per two dishes; D, mean diameter in mm; Char, character; C, clear; T, turbid; and T±, slightly turbid; *p*H was 6.9 to 7.0.

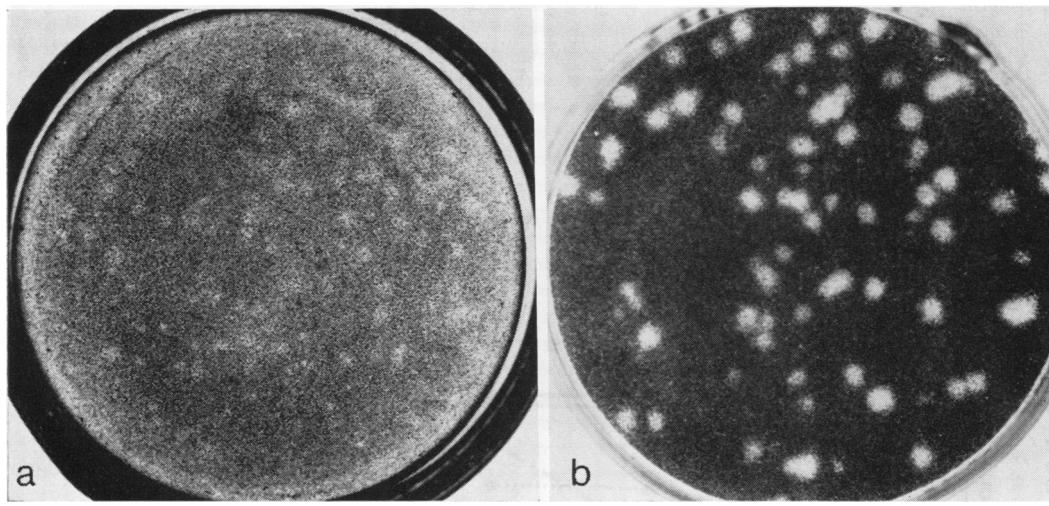

FIG. 1. *Plaques produced by virus under agar overlayer. (a) RA27/3 virus; (b) HPV-77 virus.*

To ascertain the role of agar inhibitors in determining plaque size, comparative titrations were performed with an overlayer of agar, agar plus DEAE-dextran (500 μg/ml), or carboxymethyl cellulose (CMC). Table 1 shows the results of a representative experiment in which three viruses, RA27/3, HPV-77, and H-600 (a low-passage level AGMK virus), were tested. The addition of DEAE-dextran to an agar overlayer increased the sizes of plaques formed by all three viruses. In terms of plaque number, the H-600 strain was strikingly enhanced, whereas RA27/3 showed a slight increase, and HPV-77 showed little or no change. With an overlayer of CMC, the size of the plaques produced by all three viruses was identical. HPV-77 produced no more plaques under CMC than under agar; however, RA27/3 and H-600 developed many more plaques, and their turbid character was preserved (Fig. 1a, 2a). In contrast, HPV-77 plaques were clear under both agar and CMC (Fig. 1b, 2b). The apparent increase in plaque size under CMC in Fig. 2 is the result of staining these plates at a later time postinoculation.

Under 0.25 to 0.5% agarose, which contains no agar inhibitors, all viruses produced large plaques (about 3 to 4 mm in diameter), but the large-plaque viruses still showed a slight advantage in size, and the clear or turbid character of the plaques was retained.

Influence of pH on plaque morphology. Slight variations in plaque morphology were observed in different experiments which could usually be related to accidental changes in *p*H. Therefore, the role of *p*H during incubation of small-plaque and large-plaque rubella virus variants was studied. With agar overlayers, the differences in plaque size were more pronounced when the *p*H was slightly acid. These results could be reproduced either by including different amounts of sodium bicarbonate in the overlayers or by incubation of the cell-virus mixtures in an atmosphere of 2.5 or 4% CO_2. Although HPV-77 virus was little influenced by acid *p*H, the small-plaque RA27/3 virus was inhibited.

With CMC overlayers, however, slightly acid conditions resulted in higher titers and a clearer appearance of plaques in the case of almost all

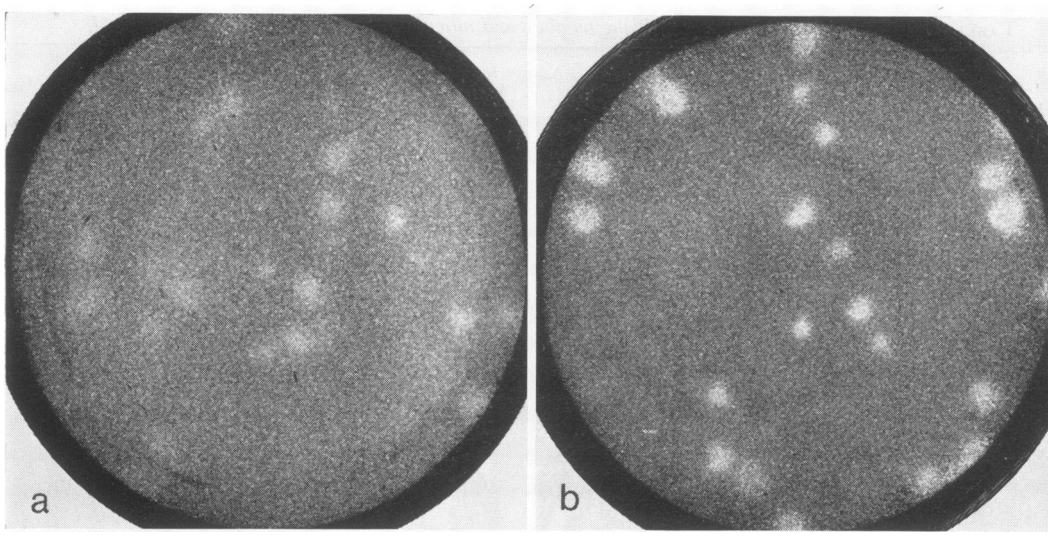

FIG. 2. *Plaques produced by virus under carboxymethyl-cellulose overlayer. (a) RA27/3 virus; (b) HPV-77 virus.*

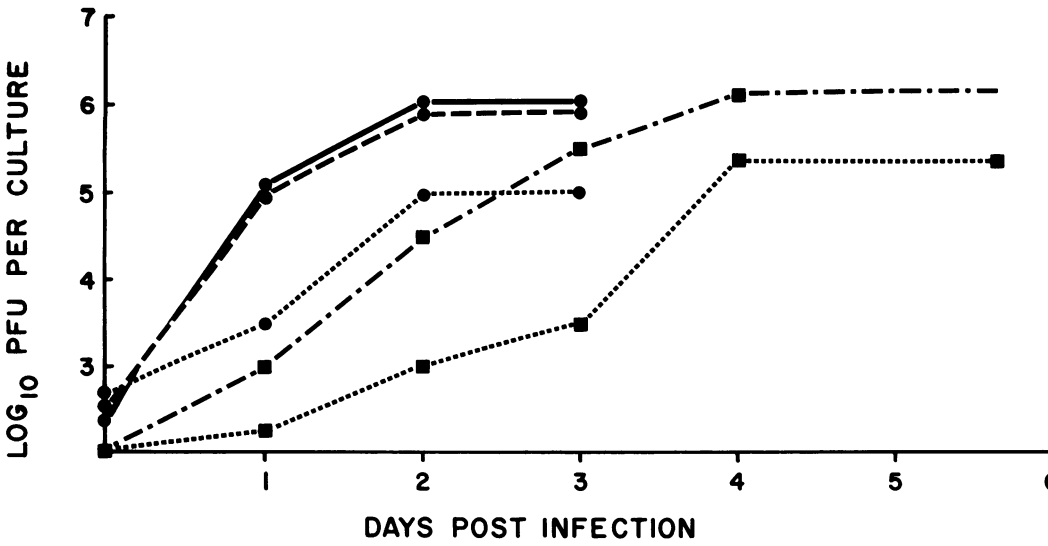

FIG. 3. *Two experiments showing the comparative growth of RA27/3, HPV-77, and West Point rubella viruses in RK_{13} cells. Experiment 1: inocula, 2×10^4 PFU per culture; input multiplicity, 1:100. Symbols: ● with short dashes, RA27/3; ● with long dashes, West Point; and ● with solid bars, HPV-77. Experiment 2: inocula, 3×10^2 PFU per culture; input multiplicity, 1:10,000. Symbols: □ with short dashes, RA27/3; □ with interrupted dashes, fresh isolate.*

viruses tested. The single exception was the Cendehill strain, which was specifically inhibited in plaque size and number by acid *p*H conditions.

Relationship of plaque morphology to the host cell and influence of tylosin. Treatment of RK_{13} cells with tylosin (3) resulted in changes in morphology and metabolic activity. After tylosin treatment, the cells grew faster, produced more acid, and showed more irregular multinucleated cells than did the untreated cells. Since metabolic activity and acid production were greater in the tylosin-treated cells, the resultant tendency toward acid *p*H produced more pronounced differences in plaque appearance between small-plaque and large-plaque viruses.

Growth curves of small-plaque and large-

TABLE 2. *Kinetics of virus and interferon production in RK_{13} cells by different rubella viruses after equal multiplicities of infection (1×10^3 PFU/plate)*

Virus strain	Growth tissue	Virus titers (log_{10} PFU/ plate) on day					Interferon titers on day[a]				
		1	2	3	4	5	1	2	3	4	5
HPV-77	RK_{13}	4.5	5.7	6.0	6.0	5.5	0	16	64	256	128
West Point	RK_{13}	4.0	5.5	5.9	6.0	5.4	0	8	64	256	64
H-600	GMK	4.2	5.3	5.7	6.2	5.3	0	4	16	256	64
RA27/3	RK_{13}	3.0	4.0	5.0	5.0	4.5	0	4	16	256	64
RA27/3	WI-38	<3.0	4.7	4.7	ND	ND	0	2	8	64	32

[a] Reciprocal of dilution, giving 50% reduction of VSV plaques.

plaque viruses in RK_{13} cells. A comparison was made of the multistep growth curves of the small-plaque RA27/3 virus and the large-plaque West Point and HPV-77 viruses in RK_{13} cells. Confluent monolayers of RK_{13} cells were infected with equal multiplicities of the three viruses, as described in Materials and Methods. The titers of virus released into the extracellular fluid after infection are shown in Fig. 3, experiment 1. At each point tested, the titer of RA27/3 virus was about 1 log_{10} $TCID_{50}$ lower than that produced by the West Point or HPV-77 viruses. Figure 3 shows the results of another growth curve experiment (experiment 2) in which RA27/3 virus was compared to three freshly isolated low-passage level rubella viruses passed in AGMK cells. Although the multiplicity of infection was lower than in experiment 1, RA27/3 virus was again distinguished by its slower growth and lower peak titers.

Interferon production by small-plaque and large-plaque viruses. Samples obtained from growth curve experiments were tested for the presence of interferon. All the rubella virus strains tested produced a substance which resembled interferon in acid resistance, nonsedimentability sensitivity to trypsin, and inhibition of VSV.

Table 2 summarizes the kinetics of virus and interferon production by five representative rubella virus variants inoculated on RK_{13} cell cultures in approximately equal virus-cell multiplicities. The data led to several conclusions. First, the results confirmed earlier demonstrations that the RA27/3 virus synthesizes less virus in RK_{13} cells. Second, the three small-plaque viruses, H-600, RA27/3 passed in RK_{13} cells, and RA27/3 passed in WI-38 cells, produced the same or slightly lower titers of interferon as did the two large-plaque variants. Third, although the RA27/3 virus produced about the same amount of interferon as the freshly isolated H-600 virus, this amount of interferon was proportional to a smaller amount of virus synthesis.

During these experiments, RK_{13} cells maintained in vitro for several days became significantly less susceptible to superinfection with VSV: sensitivity of such cells declined as much as 10-fold. Cells treated with interferon 48 hr after being placed in Petri dishes showed the highest sensitivity to VSV.

Antigenic analysis of rubella virus variants. For plaque reduction tests, a CMC overlayer with 0.11% sodium bicarbonate was found the most satisfactory, since titrations of the same virus pools varied within only 0.3 log_{10} PFU.

Antisera were prepared against three virus strains: RA27/3, pool C (10^7 PFU/ml); HPV-77 ($10^{6.5}$ PFU/ml); and West Point ($10^{6.5}$ PFU/ml). Serial fourfold dilutions of antisera were made in medium 199 supplemented with 20% normal rabbit serum, and equal volumes of the respective viruses were added in a concentration of 30 to 50 PFU/0.2 ml.

Table 3 presents the results of cross-neutralization tests of RA27/3, West Point, and HPV-77 viruses with their antisera. Specificity was greatest in the antiserum prepared against HPV-77 virus, since it neutralized the homologous virus to the highest degree. On the other hand, antiserum against the West Point virus neutralized all three variants equally. Antiserum against RA27/3 neutralized both RA27/3 and HPV-77 but was less active against West Point virus. Similar neutralization patterns were observed in four of five antisera produced against RA27/3 and in both antisera produced against HPV-77.

To enhance differences in neutralization between homologous and heterologous viruses, absorption tests were carried out with cobalt chloride-precipitated antigens. West Point virus grown in RK_{13} cells without serum was used for absorption. The precipitate obtained from 10 ml of virus harvest was used to absorb 1 ml of antiserum. As a control, precipitate produced by cobalt chloride treatment of medium 199 without virus was also used to absorb the antiserum. After

TABLE 3. *Neutralization of three strains of rubella virus by homologous and heterologous rabbit antisera*

Antiserum against	Virus		
	RA27/3	West Point	HPV-77
RA27/3	28[a]	10	40
West Point	28	16	40
HPV-77	6	5	20

[a] Reciprocal of serum dilution giving 70% reduction of PFU.

TABLE 4. *Neutralization of West Point and RA27/3 viruses by an antiserum prepared against RA27/3 before and after absorption of the serum with West Point virus*

Experiment no.	Unabsorbed antiserum		Absorbed antiserum	
	RA27/3	West Point	RA27/3	West Point
1	32[a]	16	32	4
2	20	10	20	5
3	16	16	16	8

[a] Reciprocal of serum dilution giving 70% reduction of PFU.

inactivation for 30 min at 56 C, the absorbed antisera were titrated for neutralizing activity against RA27/3 and West Point viruses in the presence of 20% normal rabbit serum (Table 4). Although absorption of RA27/3 antiserum with West Point virus slightly reduced the titer of the antiserum against both viruses, it enhanced the differences in neutralizing activity. Further work using absorbed antisera is in progress.

DISCUSSION

The results of this study show that rubella virus variants cultured in RK_{13} cells can be differentiated by plaque morphology and other characteristics.

The in vitro properties of rubella viruses have been reported to change as a result of tissue culture passage. Parkman et al. (9) observed that their high-passage level AGMK vaccine strain differed from the low-passage level virus in the ability to produce large plaques in RK_{13} cells and more interferon in AGMK cells. Huygelen and Peetermans (5) found that passage of Cendehill strain virus in primary rabbit kidney cells resulted in a loss of antigenicity when inoculated in rabbits. Vaheri et al. (18) reported that passage of RA27/3 virus in BHK21 cells resulted in the deadaptation of the virus to growth in AGMK cells.

In these studies, RA27/3 virus plaques were small and turbid. The turbidity of the plaques reflects the failure of RA27/3 and other small-plaque viruses to cause complete lysis of cells in a plaque. It should be stressed that the appearance of rubella virus plaques in RK_{13} cells is dependent on the preservation of the cells as well as on the virus strain used. If the maintenance of the cell sheet is good, plaques may even show intensively stained central areas, giving the appearance of the red plaques that have been described for a number of viruses (17). However, if cell maintenance is poor, the distinctions between turbid and clear plaques are blurred.

Agar inhibitors have an important effect on plaque size. The size of plaques formed by rubella virus variants in the absence of agar inhibitors (whether grown in agar treated with DEAE-dextran, in CMC, or in agarose) was similar. The characteristic variations in the appearance of plaques, however, remained the same.

The cell culture in which the virus being tested has been passaged probably has an effect on the appearance of rubella plaques in RK_{13} cells. We have passed HPV-77 virus in BHK21 cells and found a reduction in plaque size. M. Butler has also reported a decrease in the size of rubella plaques after passage in WI-38 cells (Proc. Soc. Gen. Microbiol, p. viii, 1966). However, the inherent genetic characteristics of the strain may be more important, since G. D. Lawrence and J. Gould have found strains isolated from rubella-syndrome infants which form large plaques similar to those produced by high-passage laboratory strains (Proc. 23rd Symp. Microbiol. Stand. Rubella Vaccines, *in press*).

The possibility of antigenic differentiation among rubella viruses requires more detailed analysis. The greater neutralization of HPV-77, not only by antisera prepared against HPV-77 itself but also by antisera against two other viruses, resembled the antibody-sensitive virus described for Coxsackie B4 strains (2) or the B phase of ECHO 6 strains (6). These antigenic differences, however, must be reinvestigated with monodispersed virus, in view of a recent report on the influence of virus aggregation on neutralization (19). On the other hand, the fact that RA27/3 virus was neutralized to a greater extent by homologous antiserum than by antiserum prepared against HPV-77 suggests that real antigenic differences exist in rubella viruses.

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